

HYBRID SHIGA-LIKE TOXIN ~~HYBRID TOXINS COMPRISING SHIGA OR SHIGA-LIKE TOXIN SUBUNITS FUSED TO ESCHERICHIA COLI HEAT LABILE ENTEROTOXIN SUBUNITS AND VACCINES THEREOF~~

5 The present invention relates to a hybrid bacterial toxin subunit, to a hybrid bipartite bacterial toxin and to nucleic acid molecules comprising a nucleotide sequence encoding such bacterial toxins. Furthermore, the invention relates to vaccines comprising said bacterial toxins and to their use in vaccines, to methods for the preparation of such vaccines and to the use of such bacterial toxins for the
10 manufacture of such vaccines.

BACKGROUND OF THE INVENTION

Many members of the Enterobacteriaceae such as *Shigella* and *Escherichia coli* are known to produce one or more toxins. Amongst these are several potent cytotoxins and neurotoxins. *Shigella dysenteriae* is known to produce the so-called Shiga-toxin
15 (Sandvig, K., Toxicon 39: 1629-1635 (2001)). A group of very closely related *Escherichia coli* toxins is toxic to African green monkey (vero) cells, and thus they became known as verotoxins. These toxins show a close resemblance to a cytotoxic toxin that was earlier found in *Shigella dysenteriae* type 1, which explains their currently used name: Shiga-like toxins (SLT). The Shiga-like toxins have been
20 described i.a. in a review by Agbodaze, D. (Comp. Immunol., Microbiol. & infectious diseases 22: 221-230 (1999)) and in a review by O'Brian, .D. and Holmes, R.K. (Microbiol. Review 51: 206-220 (1987)).

It goes without saying that the invention is applicable to both the Shiga-toxin and the
25 Shiga-like toxins. Shiga-like toxins are now known to be the cause of i.a. hemorrhagic colitis and hemolytic-uremic syndrome in humans (Karmali et al., Lancet I: 1299-1300 (1983)), diarrhoea in calves (Chanter, N., Vet. Microbiol. 12: 241-253 (1986) and Mainil et al., Am. J. Vet. Res. 48: 734-748 (1987)) and edema disease in swine (Dobrescu, L., Am. J. Vet. Res. 44: 31-34 (1983), Gannon, V.P.J. et al., Can. J. Vet.
30 Res. 53: 306-312 (1989), Marques, L. R. M., et al., FEMS Microbiol. Letters 44: 33-

38 (1987), Smith, H. W. et al., J. Gen. Microbiol. 129: 3121-3137 (1983) and Smith, H.W. et al., J. Med. Microbiol. 1: 45-59 (1968)).

Clinical manifestations of edema in pigs, i.a. neurological dysfunction, result from
5 microangiopathy and vascular necrosis caused by a specific Shiga-like toxin variant
Stx2e (Nielsen, N. O., Edema Disease, p. 528-540 (1986) *In* A. D. Lehman, Straw, B.,
Glock R.D. et al. (ed.), Diseases of swine, 6th ed. Iowa State University Press, Ames.
USA), (Gannon, V.P.J. et al., Can. J. Vet. Res. 53: 306-312 (1989), Kurtz, H.J. et al.,
Am. J. Vet. Res. 30: 791-806 (1969) and Marques, L. R. M., et al., FEMS Microbiol.
10 Letters 44: 33-38 (1987)). This variant Stx2e, also known in the art as SLT-IIe, SLT-
IIv, Verocytotoxin 2e and VT2e, causes a disease that strikes approximately one week
following weaning. The disease, characterised by the edema and the subsequent
specific neurological disturbances that it causes, is generally known as post-weaning
edema (PWE) or edema disease.

15

Shiga-toxin and all Shiga-like toxins share the same general structure. They consist of
a single A-subunit bound to multiple copies of a B-subunit. Normally, a single A-
subunit is bound to a pentamer of B-subunits. The A-subunit is the actual toxin-part: it
plays a role in the inhibition of the host's protein synthesis. The B-subunit, more
20 specifically when in its pentamer form, is associated with receptor binding. A single
B-subunit is about 7.5 kD, whereas the A-subunit is about 32 kD.

The DNA-sequence of the A1-part (see below) of the Shiga-like toxin variant Stx2e is
provided in SEQ ID NO: 1. ~~The full sequence of many other Shiga-like toxin~~
25 ~~variants can easily be found at the website of the National Center for~~
~~Biotechnology Information, [www.NCBI.NLM.NIH.GOV](http://www.ncbi.nlm.nih.gov). The search strategy is~~
~~known to the skilled person, but merely as an example, in the nucleotide bank it~~
~~suffices to fill in "shiga like toxin" as search terms to find all known variants and~~
~~their description. Alternatively, it is possible to simply use the sequence of the~~
30 ~~A1-part of the Shiga-like toxin of SEQ ID NO: 1 and blast it against the bank of~~

~~bacterial genes of the website of the National Center for Biotechnology Information. This will equally provide other known Shiga-like toxin variants.~~

Figure 1: shows a schematic drawing of a typical Shiga-like toxin; its overall structure,
5 the location of the A1/2 parts (see below) of the A-subunit and the location of the B-subunits.

The whole toxin is therefore best described as a bipartite toxin (i.e.: a toxin consisting of two parts) comprising a single A-subunit and single pentamer formed by 5 B-subunits. The A-subunit as such can subsequently be functionally divided into an A1-
10 part being the actual enzymatic part, and an A2-part being the part of the A-subunit involved in binding to the pentamer of B-subunits. The binding of the A-subunit, through the A2-part of the A-subunit, to the B-subunit follows the lock-and-key principle: the A2-part of the A-subunit of Shiga-like toxin only fits into the B-subunit
15 of Shiga-like toxin, and not to other, though closely related, B-subunits such as e.g. the B-subunit of the Heat-labile enterotoxin (LT) of Escherichia coli.

It is known that vaccination with inactivated toxins can be used to prevent disease caused by Shiga-like toxin producing E. coli strains. (Awad-Masalmeh, M., In Proc of
20 the 10th Int. Pig Vet. Soc. Congress, Rio de Janeiro, Brazil (1988), Awad-Masalmeh, M., Dtsch. Tieraertzl. Wochenschr. 96: 419-421 (1989), Howard, J.G., Br. J. Exp. Pathol. 36: 439-4476 (1955), Islam, M.S., and Stimson, W.H., J. Clin. Lab. Immunol. 33: 11-16 (1990), MacLeod, D.L and Gyles, D.L., Vet. Microbiol. 29: 309-318 (1991), Wadolkowsky, E.A. et al., Infect. & Immun. 58: 3959-3965 (1990), Bosworth,
25 B.T. Infect. & Immun. 64: 55-60 (1996)).

The genomic organisation as well as the location and sequence of the genes encoding the A- and B-subunits for Shiga-like toxins is known (Spicer E.K. et al., J. Biol. Chem., 257:5716-5721 (1982), Calderwood, S.B. et al., Proc. Natl. Acad. Sci. USA
30 84: 4364-4368 (1987), Dallas W.S. and Falkow S., Nature 288: 499-501 (1980), Leong J. et al., Infect. Immun. 48: 73-77 (1985)).

Therefore, in principle, having the necessary genetic information at hand, and knowing that vaccination with inactivated toxins can be used to prevent disease caused by Shiga-like toxin producing E. coli strains, large-scale in vitro expression of the genes encoding the A- and B-subunits seems a good starting point for vaccine production.

Against expectations however, although very efficient for the production and subsequent purification of both the A- and B-subunit of the comparable Heat-labile enterotoxin (LT) of Escherichia coli (see below), expression/purification turned out to be very difficult for Shiga-toxin and Shiga-like toxins.

First of all, although expression of the Shiga-like toxin B-subunit in a bacterial expression system is not a problem (Acheson et al., Infect. & Immun. 63: 301-308 (1995)), the Shiga-like toxin A-subunit can not, or only in minute quantities be expressed in bacterial expression systems.

Moreover, purification of the bipartite Shiga-toxin and Shiga-like toxin (contrary to the purification of LT) is both difficult and expensive. PCT-patent application WO 98/54215 provides ways of overcoming the difficulties experienced with purification, but relies therefore upon the use of affinity columns using expensive affinity ligands comprising disaccharides. For the preparation of a Shiga-toxin or Shiga-like toxin-based vaccine, this method of purification is from an economical point of view less desirable.

Therefore, both the expression and the purification of a Shiga-toxin or Shiga-like toxin remain problematic.

BRIEF SUMMARY OF THE INVENTION

It is an objective of the present invention to provide novel hybrid Shiga-toxin and Shiga-like toxins that do not suffer from the problems identified above.

Such novel hybrid Shiga-toxins and Shiga-like toxins differ from the known Shiga-like toxins in that they comprise the A1-part of the Shiga-like toxin, that is fused to the A2-part of the heat-labile enterotoxin (LT) of Escherichia coli. In the wild-type situation, the A1-part of the Shiga-like toxin is fused to the A2-part of the Shiga-like toxin.

It was surprisingly found now, that this hybrid Shiga- or Shiga-like A-subunit, contrary to its natural counterpart, can efficiently be expressed in bacterial expression systems. Also, it can easily and by inexpensive methods be purified. Moreover, this hybrid Shiga- or Shiga-like subunit comprising the A1-part of Shiga- or Shiga-like toxin but now fused to the A2-part of the LT is, even more surprisingly, fully capable of inducing protection against the wild-type Shiga- or Shiga-like toxin.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Schematic drawing of a typical Shiga-like toxin; its overall structure, the location of the A1/2 parts of the A-subunit and the location of the B-subunits are shown.

Figure 2: Construction of pMMB Stx2eA₁LTA₂B

Figure 3: Western blot developed with anti LT serum

Lane 1: Stx2eA₁LTA₂B; lane 2: LTA/B; lane 3: prestained marker

Figure 4: Galactose-silica purification. PAAGE-gel, coomasie-stained.

Lane 1: prestained marker; lane 2: whole culture pMMB Stx2eA₁LTA₂B after induction; lane 3: non bound fraction; lane 4: wash volume 1; lane 5: wash volume 5; lane 6 : purified Stx2eA₁LTA₂B eluate 1; lane 7 : purified Stx2eA₁LTA₂B eluate 2; lane 8 : purified Stx2eA₁LTA₂B eluate 3; lane 9 : purified Stx2eA₁LTA₂B eluate 4; lane 10 : purified Stx2eA₁LTA₂B eluate 5; lane 11 : purified Stx2eA₁LTA₂B eluate 6; lane 12 : purified Stx2eA₁LTA₂B eluate 7 lane 13 : purified Stx2eA₁LTA₂B eluate 8

DETAILED DESCRIPTION OF THE INVENTION

- Heat-labile enterotoxin (LT) of *Escherichia coli*, like the Shiga-like toxin of
- 5 *Escherichia coli*, is a bacterial protein toxin with a AB5 multimer structure, in which the B pentamer has a membrane binding function and the A subunit is needed for enzymatic activity (Fukuta, S. et al., Inf. & Immun. 56: 1748-1753 (1988), Pickett, C.L. et al., J. Bacteriol. 165: 348-352 (1986), Okamoto, K. et al., J. Bacteriol. 180: 1368-1374 (1998) and Lea, N. et al., Microbiology 145: 999-004 (1999)).
- 10 The expression “fused to” means that the amino acid sequence constituting the A1-part is covalently bound to the amino acid sequence constituting the A2-part. This means that the final subunit forms a single protein, as is the case in the wild-type situation.
- 15 Therefore, one embodiment of the present invention relates to a hybrid bacterial toxin A-subunit that comprises an A1-part of Shiga-like toxin fused to an A2-part of *Escherichia coli* heat-labile enterotoxin (LT).
- 20 The boundaries of the A1- and the A2-part of the A-subunit can be drawn quite precise for both the Shiga-like toxin and for LT. The A1- and A2-part are bound together by a short loop between two disulphide-linked cysteines. It is this loop that connects the A1-part and the A2-part. After entrance of the LT or Shiga-like toxin in the mammalian cell, a cleavage occurs in this loop, during which the (in the case of
- 25 Shiga-like toxin 27.5 kD) A1-part and (in the case of Shiga-like toxin 4.5 kD) A2-part become separated (Okamoto, K. et al., J. Bacteriol. 180: 1368-1374 (1998) and Lea, N. et al., Microbiology 145: 999-004 (1999)).
- In the sequence as depicted in SEQ ID NO: 1 an example of the nucleic acid sequence
- 30 of a hybrid A-subunit according to the invention comprising the Stx2e A1 part and the

LT-A2-part is shown. The amino acid sequence of the hybrid bacterial toxin encoded by this sequence is depicted in SEQ ID NO. 2.

The nucleic acid sequence encoding the hybrid A-subunit starts at position 1 and stops at position 951. In this example, the Stx2e A1-part of the A subunit starts at nucleic acid position 1 and ends at position 789, and thus just upstream the first of the disulphide-linked cysteines. The LT-A2-part of the A subunit starts at nucleic acid position 790 and ends at position 951.

The disulphide-linked cysteine residues are coded for at respectively positions 790-792 and 826-828.

10 The A1-part can therefore also be referred to as the part that is located at the N-terminal side of the loop, whereas the A2-part can be referred to as the part that is located at the C-terminal side of the loop.

It is clear that in principle the transition point between the A1-part and the A2-part is not critical. In the example given above, the transition point is located just upstream the first of disulphide-linked cysteines. It could however equally well be located somewhere between the two cysteine residues or just downstream of the second disulphide-linked cysteine at position 826-828. Actually, there is only one prerequisite: the A1-part must be capable to provide immunity against the Shiga-like toxin and the A2-part must be capable of binding to the LT-pentamer. Even more, there is no need to maintain, in the hybrid A-subunit according to the invention, the proteolytic cleavage site in the A-subunit, since this plays no role in the induction of immunity.

25 Additionally, it is shown in SEQ ID No: 3 where the LT-B subunit is located. The nucleotide sequence encoding this subunit starts at position 951 and ends at position 1322. Of course it is beneficial to have the nucleotide sequences encoding the hybrid A-subunit according to the invention and the LT-B-subunit at one and the same plasmid, as is the case in this example. Such a plasmid provides at the same time the genetic information for both the A- and the B-subunit of the bipartite bacterial toxin according to the invention.

30

The coding sequences can be brought under the control of one and the same promoter, as is the case in SEQ ID No: 1. But if further fine-tuning of the ratio hybrid A-subunit versus LT-B-subunit is required, it could be beneficial to bring the expression of both under the control of two different promoters.

5

The invention is applicable to Shiga-toxin and all Shiga-like toxin variants. These variants include those found to cause disease in humans as well as those causing disease in animals as is described above.

Since it is known that the Shiga-like toxin variant Stx2e causes post-weaning disease
10 in pigs, this variant clearly is an attractive candidate for use in vaccines for pig industry. Thus, a preferred form of this embodiment relates to hybrid A-subunits in which the A1-part of the A-subunit is an A1-part of Stx2e.

Especially beneficial is the expression of the hybrid toxin A-subunit according to the
15 invention in the presence of the gene encoding the B-subunit of the heat-labile enterotoxin. This was already mentioned above. Expression of both the hybrid A-subunit hybrid Shiga-like toxin according to the invention and the heat-labile enterotoxin in the same cell leads to spontaneous formation of the hybrid bipartite bacterial toxin, i.e. a toxin having the A1-part of Shiga-like toxin fused to the A2-part
20 of LT, and bound to the B-subunit of LT.

The hybrid bipartite toxin so made can first of all be easily expressed, secondly has the immunogenic properties of Shiga-like toxin, in the sense that it can be used for the induction of protection against the Shiga-like toxin, and thirdly has the advantage that
25 it can easily be purified according to methods known for the purification of LT (Uesaka, Y., et al., Microb. Pathog. 16: 71-76 (1994)).

Therefore, a more preferred form of this embodiment relates to a hybrid bipartite bacterial toxin comprising five B-subunits of Escherichia coli heat-labile enterotoxin
30 (LT) and the hybrid bacterial toxin A-subunit according to the invention.

It is clear that, because the nucleotide sequences of the genes encoding the A-subunits and B-subunits of both Shiga-like toxin and LT are known, standard techniques for genetic engineering suffice to construct a nucleotide sequence encoding the hybrid toxin subunit A according to the invention. One way of engineering such a nucleotide
5 sequence is given in the Examples. Man skilled in the art finds sufficient guidance, if necessary at all, in this Example to make comparable nucleotide sequences encoding other Shiga-like toxin variants according to the invention.

Thus another embodiment of the present invention relates to a nucleic acid molecule
10 comprising a nucleotide sequence encoding a hybrid bacterial toxin subunit according to the invention.

It would be even more beneficial to additionally add to such nucleotide sequence the nucleotide sequence encoding the B-subunit of LT. Expression of such a combined
15 nucleotide sequence in a cell would lead to the simultaneous production of the hybrid toxin A-subunit according to the invention and the LT B-subunit. **This** [[His]] in turn leads to the auto-formation of the hybrid bipartite bacterial toxin according to the invention. Below it is indicated how expression of the encoded proteins can in practice be effectuated.

20

Although efficient, it is however not necessary for the genetic information encoding the hybrid A-subunit and the B-subunit to be on the same nucleotide sequence. Merely as an example; the genetic information for each of the two subunits could be located on its own plasmid. A host cell comprising both plasmids would be capable to
25 form the hybrid bipartite bacterial protein according to the invention. It is even possible to synthesize both subunits in different bacteria, to isolate them and to bring them together under renaturing conditions after isolation.

Expression of the hybrid bacterial toxin subunit can e.g. be done by using
30 commercially available expression systems.

Therefore, in a more preferred form of this embodiment, the invention relates to DNA fragments comprising a nucleic acid molecule comprising a nucleotide sequence encoding a hybrid bacterial toxin subunit according to the invention. A DNA fragment is a stretch of nucleotides that functions as a carrier for a nucleic acid molecule

5 comprising a nucleotide sequence according to the invention. Such DNA fragments can e.g. be plasmids, into which a nucleic acid molecule comprising a nucleotide sequence encoding a hybrid bacterial toxin subunit according to the invention is cloned. Such DNA fragments are e.g. useful for enhancing the amount of DNA and for expression of a nucleic acid molecule comprising a nucleotide sequence encoding

10 a hybrid bacterial toxin subunit according to the invention, as described below.

An essential requirement for the expression of the nucleic acid molecule comprising a nucleotide sequence encoding a hybrid bacterial toxin subunit is an adequate promoter functionally linked to the nucleic acid molecule comprising that nucleotide sequence,

15 so that the nucleic acid molecule comprising the nucleotide sequence is under the control of the promoter. It is obvious to those skilled in the art that the choice of a promoter extends to any eukaryotic, prokaryotic or viral promoter capable of directing gene transcription in cells used as host cells for protein expression.

Therefore, an even more preferred form of this embodiment relates to a recombinant

20 DNA molecule comprising a DNA fragment and/or a nucleic acid molecule comprising a nucleotide sequence encoding a hybrid bacterial toxin subunit according to the invention wherein the nucleic acid molecule comprising a nucleotide sequence encoding a hybrid bacterial toxin subunit according to the invention is placed under the control of a functionally linked promoter. This can be obtained by means of e.g.

25 standard molecular biology techniques. (Maniatis/Sambrook (Sambrook, J. Molecular cloning: a laboratory manual, 1989. ISBN 0-87969-309-6).

Functionally linked promoters are promoters that are capable of controlling the transcription of the nucleic acid molecule comprising a nucleotide sequences to which they are linked.

30 Such a promoter can be the native promoter of the Shiga-like toxin or another promoter of *E. coli*, provided that that promoter is functional in the cell used for

expression. It can also be a heterologous promoter. When the host cells are bacteria, useful expression control sequences which may be used include the Trp promoter and operator (Goeddel, et al., Nucl. Acids Res., 8, 4057, 1980); the lac promoter and operator (Chang, et al., Nature, 275, 615, 1978); the outer membrane protein promoter (Nakamura, K. and Inouge, M., EMBO J., 1, 771-775, 1982); the bacteriophage lambda promoters and operators (Remaut, E. et al., Nucl. Acids Res., 11, 4677-4688, 1983); the α -amylase (B. subtilis) promoter and operator, termination sequences and other expression enhancement and control sequences compatible with the selected host cell.

10 When the host cell is yeast, useful expression control sequences include, e.g., α -mating factor. For insect cells the polyhedrin or p10 promoters of baculoviruses can be used (Smith, G.E. et al., Mol. Cell. Biol. 3, 2156-65, 1983). When the host cell is of vertebrate origin illustrative useful expression control sequences include the (human) cytomegalovirus immediate early promoter (Seed, B. et al., Nature 329, 840-842, 1987; Fynan, E.F. et al., PNAS 90, 11478-11482, 1993; Ulmer, J.B. et al., Science 259, 1745-1748, 1993), Rous sarcoma virus LTR (RSV, Gorman, C.M. et al., PNAS 79, 6777-6781, 1982; Fynan et al., supra; Ulmer et al., supra), the MPSV LTR (Stacey et al., J. Virology 50, 725-732, 1984), SV40 immediate early promoter (Sprague J. et al., J. Virology 45, 773, 1983), the SV-40 promoter (Berman, P.W. et al., Science, 222, 524-527, 1983), the metallothionein promoter (Brinster, R.L. et al., Nature 296, 39-42, 1982), the heat shock promoter (Voellmy et al., Proc. Natl. Acad. Sci. USA, 82, 4949-53, 1985), the major late promoter of Ad2 and the β -actin promoter (Tang et al., Nature 356, 152-154, 1992). The regulatory sequences may also include terminator and poly-adenylation sequences. Amongst the sequences that can be used are the well known bovine growth hormone poly-adenylation sequence, the SV40 poly-adenylation sequence, the human cytomegalovirus (hCMV) terminator and poly-adenylation sequences.

30 Bacterial, yeast, fungal, insect and vertebrate cell expression systems are very frequently used systems. Such systems are well-known in the art and generally available, e.g. commercially through Clontech Laboratories, Inc. 4030 Fabian Way,

Palo Alto, California 94303-4607, USA. Next to these expression systems, parasite-based expression systems are attractive expression systems. Such systems are e.g. described in the French Patent Application with Publication number 2 714 074, and in US NTIS Publication No US 08/043109 (Hoffman, S. and Rogers, W.: Public. Date 1
5 December 1993).

A still even more preferred form of this embodiment of the invention relates to Live Recombinant Carriers (LRCs) comprising a nucleic acid molecule comprising a nucleotide sequence encoding the hybrid bacterial toxin subunit according to the
10 invention, a DNA fragment according to the invention or a recombinant DNA molecule according to the invention. These LRCs are micro-organisms or viruses in which additional genetic information, in this case a nucleic acid molecule comprising a nucleotide sequence encoding the hybrid subunit according to the invention, has been cloned. Pigs infected with such LRCs will produce an immunological response
15 not only against the immunogens of the carrier, but also against the immunogenic parts of the protein(s) for which the genetic code is additionally cloned into the LRC, e.g. the novel hybrid bacterial toxin subunit according to the invention.

As an example of bacterial LRCs, attenuated Salmonella strains known in the art can
20 very attractively be used.

Also, live recombinant carrier parasites have i.a. been described by Vermeulen, A. N. (Int. Journ. Parasitol. 28: 1121-1130 (1998)).

Furthermore, LRC viruses may be used as a way of transporting the nucleic acid molecule comprising a nucleotide sequence into a target cell. Live recombinant carrier
25 viruses are also called vector viruses. Viruses often used as vectors are Vaccinia viruses (Panicali et al; Proc. Natl. Acad. Sci. USA, 79: 4927 (1982), Herpesviruses (E.P.A. 0473210A2), and Retroviruses (Valerio, D. et al; in Baum, S.J., Dicke, K.A., Lotzova, E. and Pluznik, D.H. (Eds.), Experimental Haematology today - 1988. Springer Verlag, New York: pp. 92-99 (1989)).

The technique of *in vivo* homologous recombination, well-known in the art, can be used to introduce a recombinant nucleic acid molecule into the genome of a bacterium, parasite or virus of choice, capable of inducing expression of the inserted nucleotide sequence according to the invention in the host animal.

5

Finally another form of this embodiment of the invention relates to a host cell comprising a nucleic acid molecule comprising a nucleotide sequence encoding a hybrid bacterial toxin subunit according to the invention, a DNA fragment comprising such a nucleic acid molecule or a recombinant DNA molecule comprising such a nucleic acid molecule under the control of a functionally linked promoter. This form also relates to a host cell containing a live recombinant carrier comprising a nucleic acid molecule comprising a nucleotide sequence encoding a hybrid bacterial toxin subunit according to the invention.

A host cell may be a cell of bacterial origin, e.g. *Escherichia coli*, *Bacillus subtilis* and *Lactobacillus* species, in combination with bacteria-based plasmids as pBR322, or bacterial expression vectors as pGEX, or with bacteriophages. The host cell may also be of eukaryotic origin, e.g. yeast-cells in combination with yeast-specific vector molecules, or higher eukaryotic cells like insect cells (Luckow et al; Bio-technology 6: 47-55 (1988)) in combination with vectors or recombinant baculoviruses, plant cells in combination with e.g. Ti-plasmid based vectors or plant viral vectors (Barton, K.A. et al; Cell 32: 1033 (1983), mammalian cells like Hela cells, Chinese Hamster Ovary cells (CHO) or Crandell Feline Kidney-cells, also with appropriate vectors or recombinant viruses.

25 Since it is now for the first time possible to make, in *in vitro* expression systems, sufficient amounts of hybrid toxin subunit A and hybrid bipartite toxin according to the invention, it becomes feasible to make vaccines based upon these hybrid toxins.

Vaccines based upon the expression products of these genes can easily be made by admixing the toxins according to the invention with a pharmaceutically acceptable carrier as described below.

30

If necessary, the toxin may be detoxified according to techniques known in the art, e.g. by formalin-treatment.

- 5 Therefore, another embodiment of the invention relates to vaccines comprising a hybrid bacterial toxin according to the invention or a hybrid bipartite bacterial toxin according to the invention, and a pharmaceutically acceptable carrier.

- 10 Another embodiment of the invention relates to the use of a hybrid bacterial toxin subunit or a hybrid bipartite bacterial toxin according to the invention for the manufacture of a vaccine for combating *Shigella* or *Escherichia coli* infection.

- 15 Alternatively, a vaccine according to the invention can comprise live recombinant carriers as described above, capable of expressing the protein according to the invention. Such vaccines, e.g. based upon a *Salmonella* carrier or a viral carrier e.g. a Herpesvirus vector have the advantage over subunit vaccines that they better mimic the natural way of infection of *Shigella* or *Escherichia coli*. Moreover, their self-propagation is an advantage since only low amounts of the recombinant carrier are necessary for immunization.

20

Vaccines can also be based upon host cells as described above, that comprise a bacterial toxin according to the invention.

- 25 Therefore, another form of the vaccine embodiment relates to vaccines comprising a live recombinant carrier according to the invention or a host cell according to the invention, and a pharmaceutically acceptable carrier.

- 30 Still another embodiment of the invention relates to the use of a live recombinant carrier or a host cell according to the invention for the manufacture of a vaccine for combating *Shigella* or *Escherichia coli* infection.

Still another embodiment of the present invention relates to the hybrid bacterial toxin subunit A or the hybrid bipartite toxin according to the invention for use in a vaccine.

5 Still another embodiment of the present invention relates to a live recombinant carrier or a host cell according to the invention for use in a vaccine.

All vaccines described above contribute to active vaccination, i.e. they trigger the host's defense system.

10 Alternatively, antibodies can be raised in e.g. rabbits or can be obtained from antibody-producing cell lines as described below. Such antibodies can then be administered to the human or animal to be protected. This method of vaccination, passive immunization, is the vaccination of choice when an animal is already infected, and there is no time to allow the natural immune response to be triggered. It is also the preferred method for vaccinating animals that are prone to sudden high infection
15 pressure. The administered antibodies against the protein according to the invention or immunogenic fragments thereof can in these cases bind directly to Shiga-like toxin. This has the advantage that it decreases or stops the damaging effects of infection with *Shigella* or *E. coli* making Shiga-like toxins.

20 Therefore, one other form of this embodiment of the invention relates to a vaccine for combating *Shigella* or *Escherichia coli* infection that comprises antibodies against the hybrid bacterial toxins according to the invention, and a pharmaceutically acceptable carrier.

25 Still another embodiment of this invention relates to antibodies against the hybrid toxins according to the invention.

Methods for large-scale production of antibodies according to the invention are also known in the art. Such methods rely on the cloning of (fragments of) the genetic information encoding the protein according to the invention in a filamentous phage
30 for phage display. Such techniques are described i.a., ~~at the "Antibody Engineering Page" under "filamentous phage display" at~~

~~marburg.de/~rek/aepphage.html, and~~ in review papers by Cortese, R. et al., (1994) in Trends Biotechn. 12: 262-267., by Clackson, T. & Wells, J.A. (1994) in Trends Biotechn. 12: 173-183, by Marks, J.D. et al., (1992) in J. Biol. Chem. 267: 16007-16010, by Winter, G. et al., (1994) in Annu. Rev. Immunol. 12: 433-455, and by
5 Little, M. et al., (1994) Biotechn. Adv. 12: 539-555. The phages are subsequently used to screen camelid expression libraries expressing camelid heavy chain antibodies. (Muyldermans, S. and Lauwereys, M., Journ. Molec. Recogn. 12: 131-140 (1999) and Ghahroudi, M.A. et al., FEBS Letters 414: 512-526 (1997)). Cells from the library that express the desired antibodies can be replicated and subsequently be
10 used for large scale expression of antibodies.

Still another embodiment relates to a method for the preparation of a vaccine according to the invention that comprises the admixing of antibodies against a hybrid bacterial toxin according to the invention and a pharmaceutically acceptable carrier.

15 An alternative and efficient way of vaccination is direct vaccination with DNA encoding the relevant antigen. Direct vaccination with DNA encoding proteins has been successful for many different proteins. (As reviewed in e.g. Donnelly et al., The Immunologist 2: 20-26 (1993)). This way of vaccination is also attractive for the
20 vaccination of humans and animals against infection with a *Shigella* or *Escherichia coli* strain producing a Shiga-like toxin.

Therefore, still other forms of this embodiment of the invention relate to vaccines comprising nucleic acid molecule comprising a nucleotide sequence encoding a
25 hybrid toxin according to the invention, DNA fragments according to the invention or recombinant DNA molecules according to the invention, and a pharmaceutically acceptable carrier.

Examples of DNA plasmids that are suitable for use in a DNA vaccine according to
30 the invention are conventional cloning or expression plasmids for bacterial, eukaryotic and yeast host cells, many of said plasmids being commercially available. Well-

known examples of such plasmids are pBR322 and pcDNA3 (Invitrogen). The DNA fragments or recombinant DNA molecules according to the invention should be able to induce protein expression of the nucleic acid molecule comprising a nucleotide sequence. The DNA fragments or recombinant DNA molecules may comprise one or
5 more nucleotide sequences according to the invention. In addition, the DNA fragments or recombinant DNA molecules may comprise other nucleic acid molecules comprising a nucleotide sequence such as the immune-stimulating oligonucleotides having unmethylated CpG di-nucleotides, or nucleotide sequences that code for other antigenic proteins or adjuvating cytokines.

10

The nucleic acid molecule comprising a nucleotide sequence according to the present invention or the DNA plasmid comprising a nucleic acid molecule comprising a nucleotide sequence according to the present invention, preferably operably linked to a transcriptional regulatory sequence, to be used in the vaccine according to the
15 invention can be naked or can be packaged in a delivery system. Suitable delivery systems are lipid vesicles, iscoms, dendromers, niosomes, polysaccharide matrices and the like, (see further below) all well-known in the art. Also very suitable as delivery system are attenuated live bacteria such as *Salmonella* species, and attenuated live viruses such as *Herpesvirus* vectors, as mentioned above.

20

DNA vaccines can e.g. easily be administered through intradermal application such as by using a needle-less injector. This way of administration delivers the DNA directly into the cells of the animal to be vaccinated. Amounts of DNA in the range between 10 pg and 1000 µg provide good results. Preferably, amounts in the microgram range
25 between 1 and 100 µg are used.

Another embodiment of the present invention relates to a nucleic acid molecule comprising a nucleotide sequence according to the invention, DNA fragments according to the invention, or recombinant DNA molecules according to the invention
30 for use in a vaccine.

Still another embodiment of the present invention relates to the use of a nucleic acid molecule comprising a nucleotide sequence, a DNA fragment or a recombinant DNA molecule according to the invention for the manufacturing of a vaccine for combating *Shigella* or *Escherichia coli* infection.

5

In a further embodiment, the vaccine according to the present invention additionally comprises one or more antigens derived from pathogenic organisms and viruses, antibodies against those antigens or genetic information encoding such antigens.

Of course, such antigens can be e.g. other *Shigella* or *Escherichia coli* antigens. It can

10

also be an antigen selected from another other pig pathogenic organism or virus.

In cases where the vaccine is used for vaccination of pigs, such organisms and viruses are preferably selected from the group of Pseudorabies virus, Porcine influenza virus,

Porcine parvo virus, Transmissible gastro-enteritis virus, Rotavirus, *Erysipelothrix rhusiopathiae*, *Bordetella bronchiseptica*, *Brachyspira hyodysenteriae*, *Shigella sp.*,

15

Salmonella choleraesuis, *Salmonella typhimurium*, *Salmonella enteritidis*,

Haemophilus parasuis, *Pasteurella multocida*, *Streptococcus suis*, *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Staphylococcus hyicus* and *Clostridium perfringens*.

20

All vaccines according to the present invention comprise a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier can be e.g. sterile water or a sterile physiological salt solution. In a more complex form the carrier can e.g. be a buffer.

25

Methods for the preparation of a vaccine comprise the admixing of a protein according to the invention and/or antibodies against that protein or an immunogenic fragment thereof, and/or a nucleic acid molecule comprising a nucleotide sequence and/or a DNA fragment, a recombinant DNA molecule, a live recombinant carrier or host cell according to the invention, and a pharmaceutically acceptable carrier.

30

Vaccines according to the present invention may in a preferred presentation also contain an immunostimulatory substance, a so-called adjuvant. Adjuvants in general comprise substances that boost the immune response of the host in a non-specific manner. A number of different adjuvants are known in the art. Examples of adjuvants frequently used in pig vaccines are muramyldipeptides, lipopolysaccharides, several glucans and glycans and **CARBOPOL** [[Carbopol]]^(R) (a homopolymer).

The vaccine may also comprise a so-called "vehicle". A vehicle is a compound to which the protein adheres, without being covalently bound to it. Such vehicles are i.a. bio-microcapsules, micro-alginates, liposomes and macrosols, all known in the art.

A special form of such a vehicle, in which the antigen is partially embedded in the vehicle, is the so-called ISCOM (EP 109.942, EP 180.564, EP 242.380)

In addition, the vaccine may comprise one or more suitable surface-active compounds or emulsifiers, e.g., **SPAN or TWEEN** [[Span or Tween]].

Often, the vaccine is mixed with stabilisers, e.g. to protect degradation-prone proteins from being degraded, to enhance the shelf-life of the vaccine, or to improve freeze-drying efficiency. Useful stabilisers are i.a. SPGA (Bovarnik et al; J. Bacteriology 59: 509 (1950)), carbohydrates e.g. sorbitol, mannitol, trehalose, starch, sucrose, dextran or glucose, proteins such as albumin or casein or degradation products thereof, and buffers, such as alkali metal phosphates.

In addition, the vaccine may be suspended in a physiologically acceptable diluent. It goes without saying, that other ways of adjuvating, adding vehicle compounds or diluents, emulsifying or stabilising a protein are also embodied in the present invention.

Vaccines based upon the bacterial toxins and/or subunits according to the invention can very suitably be administered in amounts ranging between 1 and 100 micrograms of protein per animal, although smaller doses can in principle be used. A dose exceeding 100 micrograms will, although immunologically very suitable, be less attractive for commercial reasons.

Vaccines based upon live attenuated recombinant carriers, such as the LRC-viruses and bacteria described above can be administered in much lower doses, because they multiply themselves during the infection. Therefore, very suitable amounts would range between 10^3 and 10^9 CFU/PFU for respectively bacteria and viruses.

5

Vaccines according to the invention can be administered e.g. intradermally, subcutaneously, intramuscularly, intraperitoneally, intravenously, or at mucosal surfaces such as orally or intranasally.

10 Still another embodiment of the invention relates to methods for the preparation of a vaccine according to the invention which method comprises the admixing of a hybrid bacterial toxin subunit or a hybrid bipartite bacterial toxin according to the invention and a pharmaceutically acceptable carrier.

15 Still another embodiment of the invention relates to methods for the preparation of a vaccine according to the invention which method comprises the admixing of a nucleic acid sequence, a DNA fragment or a recombinant DNA molecule according to the invention and a pharmaceutically acceptable carrier.

20 Finally, another embodiment of the invention relates to methods for the preparation of a vaccine according to the invention which method comprises the admixing of a live recombinant carrier or a host cell according to the invention or antibodies against a hybrid (bipartite) bacterial toxin according to the invention and a pharmaceutically acceptable carrier.

25

EXAMPLES

Example 1

Construction of expression plasmid

30

Bacterial strains and plasmids

E. coli host strain BL21(DE3)star, HMS174(DE3) and BL21codon+RIL(DE3) were purchased from Novagen (Madison, Wisconsin, USA). *E. coli* strain TOP10F' and plasmid pCR2.1-TOPO TA and pCR-bluntII-TOPO were purchased from Invitrogen (Groningen, the Netherlands).

- 5 Plasmid pMMB66HE has been described by Furste, J.P. et al., in Gene 48: 119-131 (1986).

PCR amplification and cloning of PCR products

PCR on *E. coli* chromosomal DNA was performed with the **SUPERTAQ PLUS**
10 **[[Supertaq plus]]** DNA polymerase. The PCR mixture contained 20 U/ml **SUPERTAQ PLUS****[[Supertaq plus]]** (HT Biotechnology Ltd, Cambridge, UK), **SUPERTAQ** **[[Supertaq]]** buffer containing (HT Biotechnology Ltd, Cambridge, UK), 8 mM dNTPs (Promega, Wisconsin, USA), 10 pmoles of primers and 15 ng chromosomal DNA of *E. coli* as DNA template. Oligonucleotide sequences of all
15 primers used for amplification of DNA are listed in table 1. PCR products were separated on agarose gel and gel purified using Qiagen PCR purification kit (Qiagen Inc., California, USA). Overlap extension PCR was performed as described in Sambrook et al. (Maniatis/Sambrook (Sambrook, J. Molecular cloning: a laboratory manual, 1989. ISBN 0-87969-309-6)). PCR products were cloned into pCR-bluntII-
20 topo using the TOPO cloning kit (Invitrogen., Groningen, the Netherlands). Cloning reactions were performed according to manufacturers instructions.

Construction of pMMB Stx2eA₁LTA₂B

Stx2eA₁ was amplified by PCR using primers #1832 and #1833 (see table 1) with
25 EDNL50 chromosomal DNA as template using high fidelity polymerase. EDNL50 was isolated from a pig diagnosed with post weaning edema disease. Any other strain producing Shiga-like toxin could for that matter have been used equally well. LTA₂LTB including the disulphide bridge was amplified using primers #1834 and #1835 (see table 1) with plasmid pMMB66-LT as template using high fidelity
30 polymerase. Again; any other strain producing LT could for that matter have been used equally well. One microliter of each PCR was used in the overlap extension PCR

product was made using primers #1832 and #1835. The obtained PCR product and pMMB66HE were digested with PstI and BamHI and subsequently ligated resulting in pMMB Stx2eA₁LTA₂B. The plasmid was checked by nucleotide sequence analysis and no artifacts were found.

5 Figure 2 shows the construction scheme of pMMB Stx2eA₁LTA₂B.

1832	AAAACTGCAGATGATGAAGTGTATATTGTTAAAGTG <u>SEQ ID NO. :5</u>
1833	GTTCTTGATGAATTTCCACAATTCAGTATAACGGCCACAG <u>SEQ ID NO.: 6</u>
1834	CTGTGGCCGTTATACTGAATTGTGGAAATTCATCAAGAAC <u>SEQ ID NO.: 7</u>
1835	TCATAATTCATCCCGAATTCTGTTATATATGTC <u>SEQ ID NO.: 8</u>

Table 1

10

Example 2

Expression and purification of Stx2eA₁LTA₂B.

Expression of recombinant protein

15 *E. coli* expression strains containing a tac promoter based expression vector were grown overnight at 37°C at 200 rpm in 5 ml TB with the appropriate antibiotics and 10 mM MgSO₄. The following morning the overnight cultures were diluted 1:100 in 5 ml TB with the appropriate antibiotics. These cultures were grown under the same conditions until an OD₆₀₀ of 0.5 was reached, measured on a **NOVASPEC II**
20 **[[NovaspecII]]** spectrophotometer (Pharmacia, Woerden, the Netherlands). At this point, the cultures were induced by the addition of IPTG to a final concentration of 1mM and followed by an additional incubation at 37°C for 3 hours. 100 µl samples were taken for analysis at the beginning and end of the final incubation and of the appropriate controls. The samples were analyzed by SDS page, followed by a
25 **COOMASSIE BRILLIANT BLUE****[[Coomassie Brilliant Blue]]** staining. The

remaining culture was centrifuged at 5,000 rpm and the pellet was stored at -20°C until further use.

Polyacrylamide gel electrophoresis and western blotting

5 SDS-PAGE was performed using 4-12% Bis-Tris gels from the NuPAGE electrophoresis system (Novex, San Diego, USA). Before separation the samples were boiled for 5 minutes with sample buffer (sample:buffer=2:1) in the presence of β -mercapto-ethanol in order to get a denatured protein profile. For the separation of non-denatured protein, sample buffer without β -mercapto-ethanol was added to the
10 samples. These samples loaded onto the gel without heating. The gels were stained with **COOMASSIE BRILLIANT BLUE** **[[Coomassie Brilliant Blue]]** or blotted onto **IMMUNOBULON-P-MEMBRANE** **[[Immobulon-P-membrane]]** (Millipore, Bedford, USA) by standard semi-dry Western blotting procedures. Rabbit anti-LT polyclonal α 0508/09HRP and rabbit anti-LT polyclonal α 0506/07
15 were raised against formaline inactivated LT. The anti LT-A monoclonal was purchased from Biotrend (Köln, Germany). LT(K8425) used as positive control was from a production batch. The LT was galactose-silica purified from culture supernatant and galactose used for elution was removed by dialysis. The final product contained 156 mg/l LT.

20

Galactose purification of expressed proteins

5 ml induced culture was sonicated (Branson sonifier, Geneva, Switzerland) at duty cycle 50% and microtip to complete lysis. The lysate was centrifuged for 5 minutes at 6,000 rpm to remove insoluble protein. The cleared supernatant was applied to a 1 ml
25 galactose-silica column. Column material was supplied by Organon (Oss, the Netherlands). This column was pre-equilibrated with 10 volumes of TEAN buffer (50mM Tris, 1mM EDTA, 3mM Na-azide, 200mM NaCl, pH 7.5). After binding of the supernatant, the column was washed with 5 volumes of TEAN buffer. Purified protein was eluted with 0.5 M galactose and stored at 4°C until further use.

30

RESULTS

Expression of Stx2eA₁LTA₂B fusion protein.

Three *E. coli* expression strains were tested for expression of the fusion protein. Construct pMMB Stx2eA₁LTA₂B was brought into B121star(DE3), HMS174(DE3) and JA221 and induced as described. Expression strain B121star(DE3) gave the highest expression level (data not shown).

Identification of Stx2eA₁LTA₂ using Western blotting

SDS-PAAGE-gels described above were blotted onto **IMMOBULON-P-MEMBRANE** [[Immobulon-P-membrane]] (Millipore, Bedford, USA) by standard semi-dry Western blotting procedures.

Rabbit anti-LT polyclonal α0506/07 to develop the blot was raised against formaline inactivated LT. LT used as positive control was purified from culture supernatant using affinity chromatography (galactose-silica).

As can be seen from figure 3, lane 2, both LT subunits reacted with the polyclonal antiserum: LTA (26 kDa) and LTB (14.1 kDa). This latter band is as expected also seen in lane 1 that contains the expression products of pMMB Stx2eA₁LTA₂B. The presence of LTA₂ fragment in Stx2eA₁LTA₂ was sufficient to obtain a clearly visible Stx2eA₁LTA₂ band in lane 1 at the expected size (35.1 kDa).

Galactose purification of Stx2eA₁LTA₂B

pMMB Stx2eA₁LTA₂B was induced as described and the Stx2eA₁LTA₂B fusion protein was purified from bacterial pellet by galactose purification. Results are shown in figure 4. This figure shows the amount and purity of the Stx2eA₁LTA₂B expression products in the various fractions of the galactose-silica column: lane 1: prestained marker; lane 2: whole culture pMMB Stx2eA₁LTA₂B after induction; lane 3: non bound fraction; lane 4: wash volume 1; lane 5: wash volume 5; lane 6 : purified Stx2eA₁LTA₂B eluate 1; lane 7 : purified Stx2eA₁LTA₂B eluate 2; lane 8 : purified Stx2eA₁LTA₂B eluate 3; lane 9 : purified Stx2eA₁LTA₂B eluate 4; lane 10 : purified Stx2eA₁LTA₂B eluate 5; lane 11 : purified Stx2eA₁LTA₂B eluate 6; lane 12 : purified Stx2eA₁LTA₂B eluate 7 lane 13 : purified Stx2eA₁LTA₂B eluate 8

~~Legend to the figures.~~

~~Figure 1: Schematic drawing of a typical Shiga-like toxin; its overall structure, the location of the A1/2 parts of the A-subunit and the location of the B-subunits are shown.~~

5

~~Figure 2: Construction of pMMB Stx2eA1LTA2B~~

~~Figure 3: Western blot developed with anti LT serum~~

10 **~~Lane 1: Stx2eA1LTA2B; lane 2: LTA/B; lane 3: prestained marker~~**

~~Figure 4: Galactose-silica purification. PAAGE-gel, coomassie-stained.~~

15 **~~Lane 1: prestained marker; lane 2: whole culture pMMB Stx2eA1LTA2B after induction; lane 3: non bound fraction; lane 4: wash volume 1; lane 5: wash volume 5; lane 6 : purified Stx2eA1LTA2B eluate 1; lane 7 : purified Stx2eA1LTA2B eluate 2; lane 8 : purified Stx2eA1LTA2B eluate 3; lane 9 : purified Stx2eA1LTA2B eluate 4; lane 10 : purified Stx2eA1LTA2B eluate 5; lane 11 : purified Stx2eA1LTA2B eluate 6; lane 12 : purified Stx2eA1LTA2B eluate 7 lane 13 : purified Stx2eA1LTA2B eluate 8~~**

Abstract

The present invention relates to a hybrid bacterial toxin subunit, to a hybrid bipartite bacterial toxin and to nucleic acid molecules comprising a nucleotide sequence
5 encoding such bacterial toxins. Furthermore, the invention relates to vaccines comprising said bacterial toxins and to their use in vaccines. Finally, the invention relates to methods for the preparation of such vaccines and to the use of such bacterial toxins for the manufacture of such vaccines.

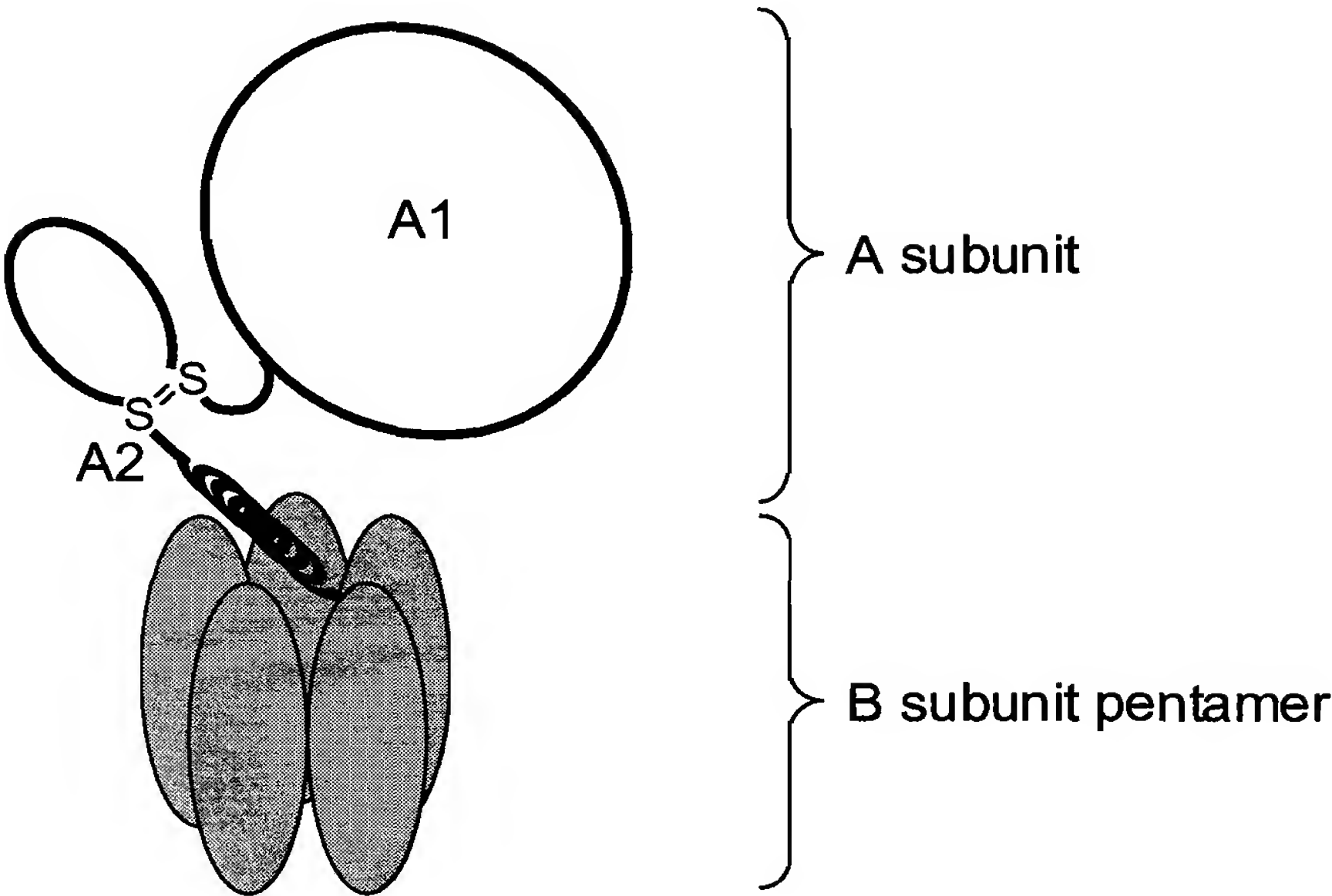


Figure 1

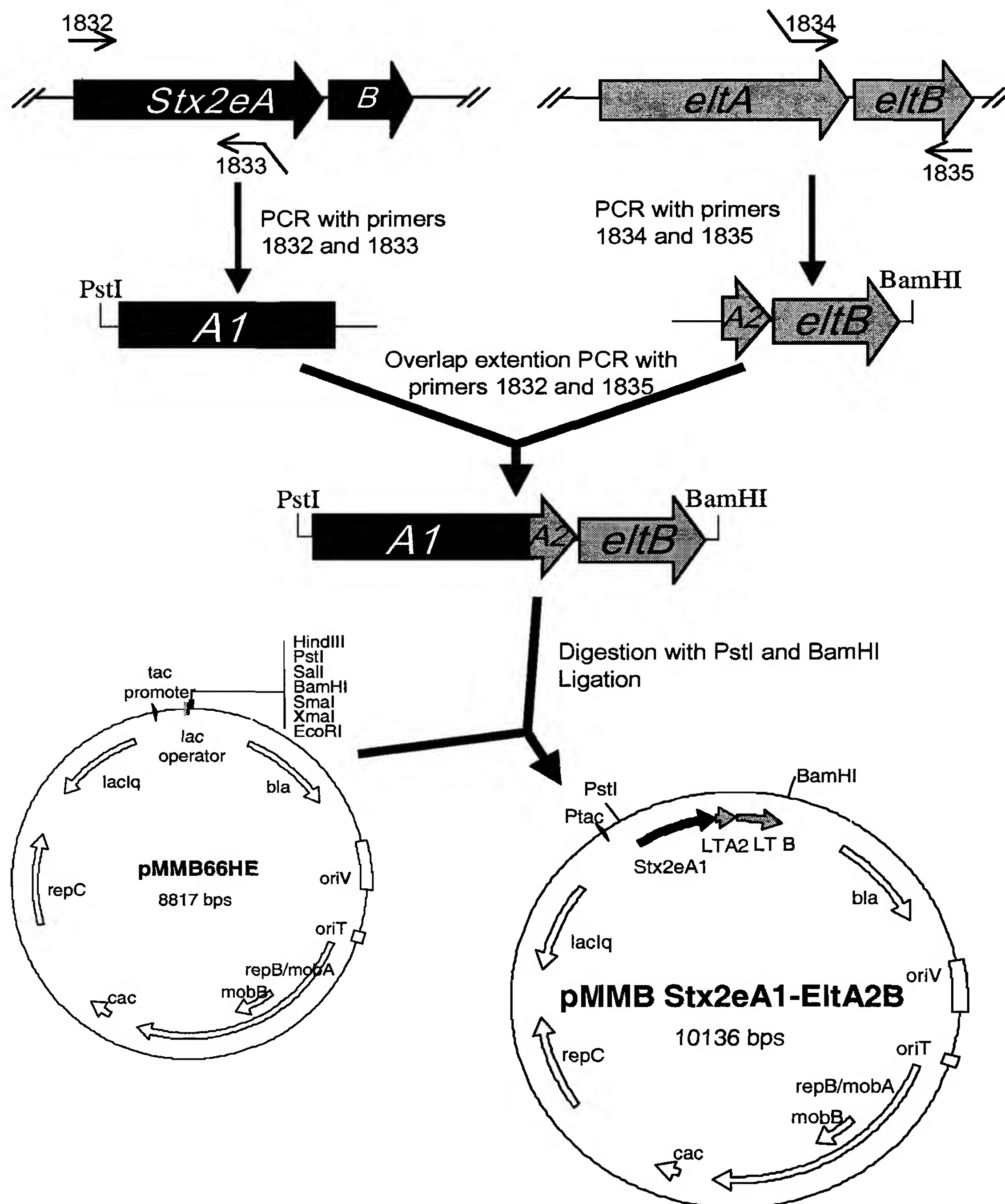


Figure 2

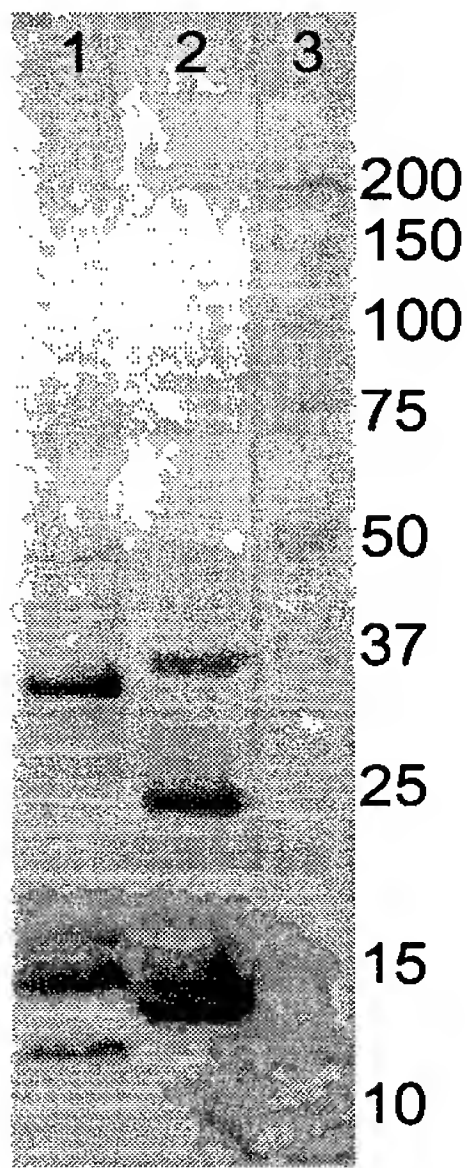


Figure 3

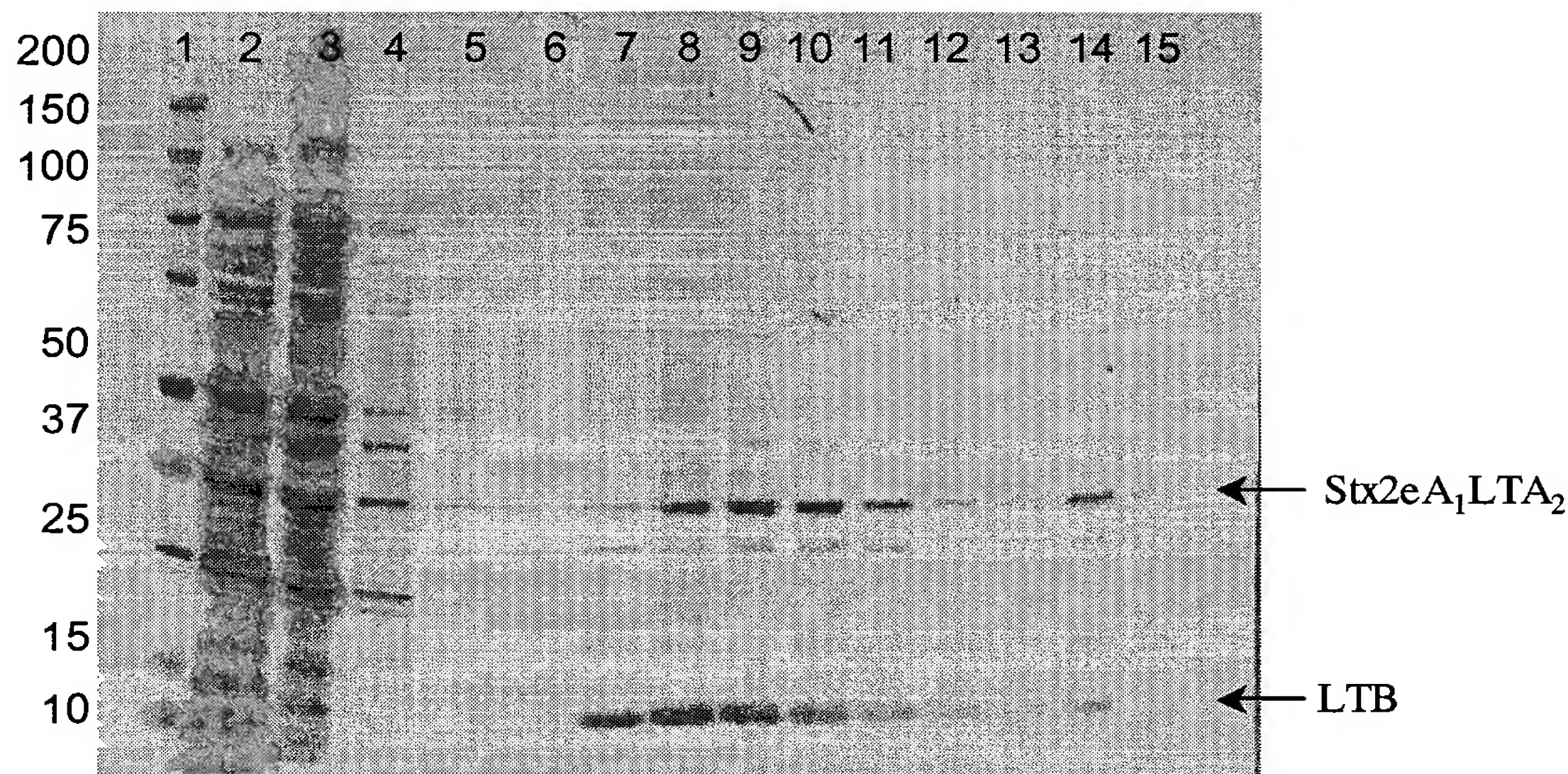


Figure 4.

SEQUENCE LISTING

<110> AKZO Nobel N.V.

<120> Shiga-like toxin vaccine

<130> 2003.006

<160> 4

<170> PatentIn version 3.2

<210> 1

<211> 1325

<212> DNA

<213> Escherichia coli

<220>

<221> CDS

<222> (1)..(954)

<400> 1

atg atg aag tgt ata ttg tta aag tgg ata ctg tgt ctg tta ctg ggt	48
Met Met Lys Cys Ile Leu Leu Lys Trp Ile Leu Cys Leu Leu Leu Gly	
1 5 10 15	

ttt tct tcg gta tcc tat tcc cag gag ttt acg ata gac ttt tcg act	96
Phe Ser Ser Val Ser Tyr Ser Gln Glu Phe Thr Ile Asp Phe Ser Thr	
20 25 30	

caa caa agt tat gta tct tcg tta aat agt ata cgg aca gtg ata tcg	144
Gln Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Val Ile Ser	
35 40 45	

acc cct ctt gaa cat ata tct cag gga gct aca tcg gta tcc gtt att	192
Thr Pro Leu Glu His Ile Ser Gln Gly Ala Thr Ser Val Ser Val Ile	
50 55 60	

aat cat aca cca cca gga agt tat att tcc gta ggt ata cga ggg ctt	240
Asn His Thr Pro Pro Gly Ser Tyr Ile Ser Val Gly Ile Arg Gly Leu	
65 70 75 80	

gat gtt tat cag gag cgt ttt gac cat ctt cgt ctg att att gaa cga	288
Asp Val Tyr Gln Glu Arg Phe Asp His Leu Arg Leu Ile Ile Glu Arg	
85 90 95	

aat aat tta tat gtg gct gga ttt gtt aat acg aca aca aat act ttc	336
Asn Asn Leu Tyr Val Ala Gly Phe Val Asn Thr Thr Thr Asn Thr Phe	
100 105 110	

tac aga ttt tca gat ttt gca cat ata tca ttg ccc ggt gtg aca act	384
Tyr Arg Phe Ser Asp Phe Ala His Ile Ser Leu Pro Gly Val Thr Thr	
115 120 125	

att tcc atg aca acg gac agc agt tat acc act ctg caa cgt gtc gca	432
Ile Ser Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala	
130 135 140	

gcg ctg gaa cgt tcc gga atg caa atc agt cgt cac tca ctg gtt tca	480
Ala Leu Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser	
145 150 155 160	

2003.006 US

Substitute Specification with mark up 12/2009

tca tat ctg gcg tta atg gag ttc agt ggt aat aca atg acc aga gat	528
Ser Tyr Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp	
165 170 175	
gca tca aga gca gtt ctg cgt ttt gtc act gtc aca gca gaa gcc tta	576
Ala Ser Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu	
180 185 190	
cgg ttc agg caa ata cag aga gaa ttt cgt ctg gca ctg tct gaa act	624
Arg Phe Arg Gln Ile Gln Arg Glu Phe Arg Leu Ala Leu Ser Glu Thr	
195 200 205	
gct cct gtt tat acg atg acg ccg gaa gac gtg gac ctc act ctg aac	672
Ala Pro Val Tyr Thr Met Thr Pro Glu Asp Val Asp Leu Thr Leu Asn	
210 215 220	
tgg ggg aga atc agc aat gtg ctt ccg gag tat cgg gga gag gct ggt	720
Trp Gly Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Ala Gly	
225 230 235 240	
gtc aga gtg ggg aga ata tcc ttt aat aat ata tca gcg ata ctt ggt	768
Val Arg Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly	
245 250 255	
act gtg gcc gtt ata ctg aat tgt gga aat tca tca aga aca atc aca	816
Thr Val Ala Val Ile Leu Asn Cys Gly Asn Ser Ser Arg Thr Ile Thr	
260 265 270	
ggt gat act tgt aat gag gag acc cag aat ctg agc aca ata tat ctc	864
Gly Asp Thr Cys Asn Glu Glu Thr Gln Asn Leu Ser Thr Ile Tyr Leu	
275 280 285	
agg gaa tat caa tca aaa gtt aag agg cag ata ttt tca gac tat cag	912
Arg Glu Tyr Gln Ser Lys Val Lys Arg Gln Ile Phe Ser Asp Tyr Gln	
290 295 300	
tca gag gtt gac ata tat aac aga att cgg gat gaa tta tga	954
Ser Glu Val Asp Ile Tyr Asn Arg Ile Arg Asp Glu Leu	
305 310 315	
ataaagtaaa atgttatgtt ttattttacgg cgttactatc ctctctatat gcacacggag	1014
ctccccagac tattacagaa ctatgttcgg aatatcgcaa cacacaaata tatacgataa	1074
atgacaagat actatcatat acggaatcga tggcaggcaa aagagaaatg gttatcatta	1134
catttaagag cggcgaaaca tttcaggtcg aagtcccgagg cagtcaacat atagactccc	1194
agaaaaaagc cattgaaagg atgaaggaca cattaagaat cacatatctg accgagacca	1254
aaattgataa attatgtgta tggaataata aaacccccaa ttcaattgcg gcaatcagta	1314
tgaaaaacta g	1325

<210> 2

<211> 317

<212> PRT

<213> Escherichia coli

<400> 2

Met Met Lys Cys Ile Leu Leu Lys Trp Ile Leu Cys Leu Leu Leu Gly

USSN 10/565,183

3

2003.006 US

Substitute Specification with mark up 12/2009

1				5						10					15
Phe	Ser	Ser	Val	Ser	Tyr	Ser	Gln	Glu	Phe	Thr	Ile	Asp	Phe	Ser	Thr
			20					25					30		
Gln	Gln	Ser	Tyr	Val	Ser	Ser	Leu	Asn	Ser	Ile	Arg	Thr	Val	Ile	Ser
		35					40					45			
Thr	Pro	Leu	Glu	His	Ile	Ser	Gln	Gly	Ala	Thr	Ser	Val	Ser	Val	Ile
	50					55					60				
Asn	His	Thr	Pro	Pro	Gly	Ser	Tyr	Ile	Ser	Val	Gly	Ile	Arg	Gly	Leu
65					70					75					80
Asp	Val	Tyr	Gln	Glu	Arg	Phe	Asp	His	Leu	Arg	Leu	Ile	Ile	Glu	Arg
				85					90					95	
Asn	Asn	Leu	Tyr	Val	Ala	Gly	Phe	Val	Asn	Thr	Thr	Thr	Asn	Thr	Phe
			100					105					110		
Tyr	Arg	Phe	Ser	Asp	Phe	Ala	His	Ile	Ser	Leu	Pro	Gly	Val	Thr	Thr
		115					120					125			
Ile	Ser	Met	Thr	Thr	Asp	Ser	Ser	Tyr	Thr	Thr	Leu	Gln	Arg	Val	Ala
	130					135					140				
Ala	Leu	Glu	Arg	Ser	Gly	Met	Gln	Ile	Ser	Arg	His	Ser	Leu	Val	Ser
145					150					155					160
Ser	Tyr	Leu	Ala	Leu	Met	Glu	Phe	Ser	Gly	Asn	Thr	Met	Thr	Arg	Asp
				165					170					175	
Ala	Ser	Arg	Ala	Val	Leu	Arg	Phe	Val	Thr	Val	Thr	Ala	Glu	Ala	Leu
			180					185					190		
Arg	Phe	Arg	Gln	Ile	Gln	Arg	Glu	Phe	Arg	Leu	Ala	Leu	Ser	Glu	Thr
		195					200					205			
Ala	Pro	Val	Tyr	Thr	Met	Thr	Pro	Glu	Asp	Val	Asp	Leu	Thr	Leu	Asn
	210					215					220				
Trp	Gly	Arg	Ile	Ser	Asn	Val	Leu	Pro	Glu	Tyr	Arg	Gly	Glu	Ala	Gly
225					230					235					240
Val	Arg	Val	Gly	Arg	Ile	Ser	Phe	Asn	Asn	Ile	Ser	Ala	Ile	Leu	Gly
				245					250					255	

4

Substitute Specification with mark up 12/2009

aaa gta aaa tgt tat gtt tta ttt acg gcg tta cta tcc tct cta tat 1004
Lys Val Lys Cys Tyr Val Leu Phe Thr Ala Leu Leu Ser Ser Leu Tyr

2003.006 US

Substitute Specification with mark up 12/2009

5	10	15	
gca cac gga gct ccc cag act att aca gaa cta tgt tcg gaa tat cgc			1052
Ala His Gly Ala Pro Gln Thr Ile Thr Glu Leu Cys Ser Glu Tyr Arg			
20	25	30	
aac aca caa ata tat acg ata aat gac aag ata cta tca tat acg gaa			1100
Asn Thr Gln Ile Tyr Thr Ile Asn Asp Lys Ile Leu Ser Tyr Thr Glu			
35	40	45	50
tcg atg gca ggc aaa aga gaa atg gtt atc att aca ttt aag agc ggc			1148
Ser Met Ala Gly Lys Arg Glu Met Val Ile Ile Thr Phe Lys Ser Gly			
	55	60	65
gaa aca ttt cag gtc gaa gtc ccg ggc agt caa cat ata gac tcc cag			1196
Glu Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp Ser Gln			
	70	75	80
aaa aaa gcc att gaa agg atg aag gac aca tta aga atc aca tat ctg			1244
Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Thr Tyr Leu			
	85	90	95
acc gag acc aaa att gat aaa tta tgt gta tgg aat aat aaa acc ccc			1292
Thr Glu Thr Lys Ile Asp Lys Leu Cys Val Trp Asn Asn Lys Thr Pro			
	100	105	110
aat tca att gcg gca atc agt atg aaa aac tag			1325
Asn Ser Ile Ala Ala Ile Ser Met Lys Asn			
115	120		
<210> 4			
<211> 124			
<212> PRT			
<213> Escherichia coli			
<400> 4			
Met Asn Lys Val Lys Cys Tyr Val Leu Phe Thr Ala Leu Leu Ser Ser			
1	5	10	15
Leu Tyr Ala His Gly Ala Pro Gln Thr Ile Thr Glu Leu Cys Ser Glu			
	20	25	30
Tyr Arg Asn Thr Gln Ile Tyr Thr Ile Asn Asp Lys Ile Leu Ser Tyr			
	35	40	45
Thr Glu Ser Met Ala Gly Lys Arg Glu Met Val Ile Ile Thr Phe Lys			
	50	55	60
Ser Gly Glu Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile Asp			
65	70	75	80
Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile Thr			
	85	90	95
Tyr Leu Thr Glu Thr Lys Ile Asp Lys Leu Cys Val Trp Asn Asn Lys			

USSN 10/565,183

6

2003.006 US

Substitute Specification with mark up 12/2009

100

105

110

Thr	Pro	Asn	Ser	Ile	Ala	Ala	Ile	Ser	Met	Lys	Asn
		115					120				